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Thesis

THE EFFECT OF HEAT UPON IMMUNE SERA

by

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(B.S. in Education, Boston University, 1944)

submitted in partial fulfilment of the
requirements for the degree of
Master of Arts

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INTRODUCTION

The purpose of this thesis is to review and discuss the studies dealing with the effect of heat upon immune sera. For the sake of completeness and in order to give the earlier workers the credit due them the early papers will be included but they will not be considered with the thoroughness devoted to some of the later papers. This procedure was decided upon because later experimenters have the advantage both of more extensive knowledge of antibodies and of more precise and better techniques in investigating them. Some of the more recent reports of experiments, in which physical and chemical denaturing agents other than heat were used, will be considered and the results compared with those obtained with heat. Some experimental work by the author will be presented.

REVIEW OF THE LITERATURE

The studies of the effect of heat on immune sera can be grouped conveniently into three periods. The first period extends roughly from the late eighteen nineties to 1910, the second from 1910 to about 1935, and the third from 1935 to the present.

Probably the first experiments to determine the effect of heat upon antibody were those of Bordet (12) in 1899. He used sera from the goat, guinea pig and rabbit that contained in

some cases normal agglutinins for V.comma and in others the immune agglutinins. A temperature of 55 degrees C had little effect, 61 to 62 degrees C weakened the agglutinins and at 70 degrees C they were inactivated. Since he found the agglutinins present in rabbit serum more resistant than those in the goat and guinea pig serum, he concluded that species differences existed with regard to the resistance of agglutinins to heat. Three years later Eisenberg and Volk (22) and Eisenberg (21) studied the effect of heat on both agglutinin and precipitin which they found to be destroyed above 60 degrees C. But they made the important observation that while both were destroyed if judged by their ability to show visible reactions such as agglutination of bacteria or precipitation of soluble antigen, the antibodies were, nevertheless, still able to combine with their specific antigens. Kraus (53) in a review, Präzipitine, stated that he and von Pirquet were the first to observe this property with bacterial precipitins when precipitating serum was heated at 50 to 60 degrees C. Later he said it was observed for other precipitins by Muller (53), Eisenberg (53), and Oppenheimer and Michaelis (53); all these investigators found that a temperature of 70 degrees C was required to destroy precipitating power and leave the power to combine.

Kraus referred, also, to papers by Tschistowitsch and Myers (53) who reported precipitins for serum and proteins wholly destroyed at 60 degrees C, and to one by Pick (53) who found bacterial precipitin destroyed at this same temperature.

Schmidt (53a), he said, had observed slower flocculation in diluted precipitating serum after it was heated one half hour at 70 degrees C. Other reviews in Kolle and Wassermann were consulted for reports of the early experiments published in foreign journals, especially the German and French.

In the article, Die Agglutination, Paltauf (64) discussed the papers dealing with the effect of heat on both normal and immune agglutinins. Rodet (64a) reported that normal and immune agglutinins for E.typhosa in rabbit serum differed in their resistance to heat, the normal being destroyed at 50 to 58 degrees C and the immune withstanding 60 to 62 degrees C without damage. Bail (64a), using normal bovine serum found its agglutinating power completely destroyed at 56 degrees C. Negrée and Raynaud (64a) in experiments with normal serum - probably human - containing agglutinins for B.melitensis, D.pneumoniae, and Staphylococci found two kinds of agglutinins present; one was specific and withstood heating to 56 degrees C, and the other, not specific, was destroyed at 56 degrees C.

Paltauf considered in varying detail the papers of Pick (64b), Rodella (64b), Widal and Sicard (64b), and Joos (64c) that dealt with the immune agglutinins. Pick (64b) reported that albumin-free immune-globulin obtained from equine antityphoid serum by repeated precipitation with salt withstood a temperature of 80 to 90 degrees C without substantial injury, and even short boiling as long as coagulation was prevented by the presence of urea. In the absence of urea coagulation occurred

at 75 C and the finely flocculant material even when dissolved immediately contained no agglutinins. These agglutinins for E. typhosa were in the pseudoglobulin of the serum. The agglutinins for V. cholera which were in the euglobulin of equine serum, Pick (64b) found were less resistant to heat with destruction occurring at temperatures over 65 C. Rodella (64b) observed that agglutinins present in Proteus antiserum withstood a temperature of 80 C; and Widal and Sicard (64b) that those present in antityphoid serum withstood a temperature of 75 C. Joos (46) studied the agglutinins for E.typhosa. He thought there were two modifications, one of which was altered at 60 to 62 C, but not the other which was neither altered at 62 C nor after one and one half hours at 65 C.

Paltauf (64) and Marrack (58) referred to the experiments of Streng,and Madsen and Streng who studied the rate of destruction of E. typhosa and E.coli agglutinins at temperatures from 65 to 75 C. In most cases the reaction representing the rate of destruction was unimolecular, but in some cases it appeared to be bimolecular. Marrack pointed out that the apparent deviations from the unimolecular type might have been due to the presence of both flagellar and somatic agglutinins.

In an article by Friedberger (28), Die bakteriziden Sera, also in Kolle and Wassermann, experiments are mentioned that dealt with the destructive effect of heat on the autolyzing power of anticholera serum. A temperature of 60 C for 20 hours

had little effect; 70 C for one hour almost wholly destroyed the antibody.

In a third article by Wassermann and Wassermann (80) it was stated that antitoxic sera began to be injured at 60 to 70 C and were very rapidly destroyed at boiling heat. Such sera when dry would withstand one half hour at 110 C and one quarter hour at 140 C.

Robert Doerr (18) in a review of the subject of allergy and anaphylaxsis stated that anaphylactic antibody was not destroyed at 56 C.

Buxton and Torrey (13) discussing the formation of agglutinoids said their experiments with antityphoid sera in agreement with those of others showed that when ordinary immune serum was heated agglutinin began to be changed to agglutinoid after 30 minutes at 65 to 75 C. They found no agglutinins left after 30 minutes at 75 C, but the agglutinoids were at their maximum. The agglutinoids in turn were considerably destroyed at 80 C and entirely at 85 C.

The view tends to persist that antibodies present in normal sera are more heat-labile than those present in immune sera. Landsteiner (56) was inclined to think this was a fact and he stated definitely that it was true with regard to the hemagglutinins. However, the experiments of Felix and Olitzki (25) with immune and normal rabbit serum both containing O agglutinins for E.typhosa did not demonstrate any difference in the way these sera were affected by heat. They reported, also, no difference in resistance to heat between normal and

immune agglutinins for B. dysenteriae. Two more recent papers, one very extensive study by Gibson (30), and the other by Jordan (47) support the view that normal agglutinins are less resistant to heat tending to be destroyed at 60 to 65 C.

Since a comparison of normal and immune agglutinins is not of major importance in this thesis the discussion will be closed with a few comments which may explain partly the discrepant results that have been observed in comparing the effect of heat upon normal and immune antibody. We know now that the same immune serum may contain more than one antibody capable of reacting specifically with a single antigen. There have been a number of papers published which establish this as a fact. Among them are those of Hooker (40, 41), Landsteiner and van der Scheer (57). We have learned that the manner in which a complex antigen is introduced, whether injected intravenously or subcutaneously influences the response of the animal (70). Another factor determining the type of antibody or antibodies present in a given serum is the length of time immunization has been carried on before bleedings are made (42). Landsteiner (56a) referred to this as a factor affecting the thermal lability of antibody, - the antibody becoming more resistant to heat as immunization was continued.

When such facts are taken into consideration it is not difficult to think that in some instances the normal and immune antibodies being compared were identical, or nearly so, and were similarly affected by heat; while in other cases the antibodies

were different enough in structure so that they were not destroyed at the same temperature.

In addition the earlier experimenters did not know about the flagellar and somatic antigens present in some of the bacteria with which they were working. Smith and Reagh (74) described these antigens in 1903. In the following year Beyer and Reagh (10) reported the different heat-lability of their respective agglutinins. Not much attention was paid to these observations until fifteen and more years later when Weil and Felix (81) published the results of their studies with the O and H antigen of Proteus X_{19} and then with the O and H antigens of the typhoid-paratyphoid group. A few years later, Orcutt (63) established the identity of the H and flagellar agglutinins and of the O and somatic. Since it has been convincingly demonstrated that these two agglutinins are dissimilarly affected by heat, and also, that only one, or both may be present in a given serum, it is possible experimenters reporting discrepant results were not working with the same antibody.

What was learned concerning the effect of heat on antibody during this first period can be summarized as follows: (1) Antibodies can be destroyed by heat, but the critical temperature is not the same for all antibodies. (2) Some antibodies are present in the pseudoglobulin fraction of serum and some in the euglobulin and these appear to differ in their thermal lability. (3) Species differences may exist with regard to the resistance of antibody to heat. (4) Normal and

immune antibodies may differ in their lability to heat. (5) Agglutinoids and precipitoids are formed when immune sera are heated. (6) Flagellar and somatic agglutinins can be differentiated by their resistance to heat. (7) Certain substances, for example, urea, that prevent the coagulation of protein will prevent destruction of agglutinins. (8) In many instances the destruction of antibody by heat occurs as a unimolecular reaction.

This first period was an exploratory one which followed soon after the recognition of antibody. Some of the experimental work was not extensive and was not adequately controlled with regard to some factors which were not known or the importance of which was not recognized. It remained for the scientists of later periods to repeat, re-interpret, and extend the experimental work dealing with the destructive or alterative effects of heat upon antibody.

During the second period extending from 1910 to about 1935 the antibodies in immune sera became important therapeutic agents in the treatment of infectious diseases. Of the therapeutic sera diphtheric antitoxin and antipneumococcal serum were studied extensively. The studies dealing with the effect of heat on antitoxin were concerned mainly with temperature during storage as a factor in loss of potency, and with heat as an effective measure in the purification of crude sera.

Anderson (1) found that diphtheric antitoxin lost 10 per cent of its potency per year when it was kept at a temperature

of 15 C. This result was confirmed by MacConkey (58). He studied, also, loss at 36 C, which he found to be 50 per cent per annum. Glenny (31) stored antitoxin at 37 C for seven years and reported a loss in potency of over 90 per cent. Marrack (59) has commented upon the fact that in the destruction of antibody by heat and in the denaturation of proteins by heat the reactions are usually of the unimolecular type. He observed that it is possible from data available to calculate the rate of destruction at different temperatures. For example, it would require "...some thousand years to destroy as much antibody at 40 C as is destroyed in one hour at 70 C". Since this did not agree with the results of Glenny mentioned above Marrack thought different processes were involved.

When Banzhaf (4) found that heating crude diphtheric antitoxin at 57 C for 12 to 15 hours resulted in the loss of only 7 per cent of the antitoxin he provided the basis of the method still used in some laboratories in purifying and concentrating diphtheric antitoxin. When the crude antitoxin was heated under these conditions the amount of serum protein precipitated by different concentrations of $(NH_4)_2SO_4$ was increased in some instances and decreased in others: 10 per cent of euglobulin increased to 41; pseudoglobulin and albumin decreased from 78 to 50 per cent and from 12 to 9 per cent respectively. With the exception of the 7 per cent which was lost, all the antitoxin was recovered in the pseudoglobulin.

Usually this purified and concentrated antitoxin flocculates

much more slowly than the crude antitoxin. Slight denaturation of the antitoxin by heat and removal of the lipids present in serum have been offered as explanations of this changed reactivity. But recent work provides a better explanation. The interesting papers of Barr (5) and Barr and Glenny (6) published more than ten years ago indicated that there was more than one antitoxin in crude serum. More recent investigations by Pappenheimer et al (66), by Kekwick and Record,(48) and Kekwick et al (49) have established definitely the presence of two antitoxins with different chemical and physical properties. These last two studies in which the electrophoretic method of analysis was used showed that the γ antitoxin present in the γ globulin was precipitated with the euglobulin, and the β antitoxin present in the β globulin was precipitated with the pseudoglobulin. The former, a rapidly flocculating antitoxin is lost in the usual methods of concentration, so that concentrated antitoxin consists of the more slowly flocculating β antitoxin.

In 1934 Gerlough and White (29) studied the effect of heat on crude tetanal antitoxin under conditions differing with regard to pH, concentration of phenol, and concentration of neutral salts. Since the minimum rate of destruction under various conditions occurred at pH 6.6 to 7.3, they concluded the process, by which the antitoxin was destroyed, was a hydrolytic one catalyzed by H^+ and OH^-. Phenol increased the rate markedly, a concentration of 0.5 per cent causing an increase of approximately 28 times. In the presence of neutral salts the

the rate was at a maximum at a total ionic strength between 0.6 and 0.9M, but when the amount of neutral salts was increased, the rate decreased. Plasma was adjusted to pH 6.7 or 6.8 and the rate of destruction determined at 60 to 65 C. At 60 C the reaction followed a unimolecular course reasonably well, but at 65 C it did not do so in the later stages of the reaction.

Hartley (35) has referred to the experiments of Madsen and Walbam with diphtheric antitoxin in which they determined the rate of destruction at 65 to 74 C. These authors, he said, concluded that the reaction representing loss of potency was a bimolecular one.

During this period there were many studies dealing with the flagellar and somatic antigens and their corresponding agglutinins. According to Topley and Wilson (75) the first reports of the existence of these two agglutinins received little attention until Weil and Felix published their papers. Arkwright (2) has referred to and discussed these in a review. He says that they found the O agglutinin was destroyed in 30 minutes at 70 C while the H agglutinin was only partly destroyed at this temperature. Orcutt (63) confirmed the work of Beyer and Reagh (10) with hog-cholera bacillus in finding the O or somatic agglutinins destroyed at 70 C while the flagellar agglutinins were not appreciably affected at this temperature. After being heated at 75 C, the latter reacted more slowly.

Felix and Olitzki (25) reported that when agglutinating sera were diluted before they were heated, the loss in potency

was less in the more dilute samples. With regard to the effect of heat on O and H agglutinins their results were in agreement with those of other workers. They said the agglutinins for B.melitensis had the same thermal lability as the O agglutinins, but those for the Flexner and Y strains of the Shigella were more resistant than the O agglutinins but much less so than the H agglutinins. It is interesting that they found the O agglutinin for Proteus X_{19} in the serum, of patients with typhus less resistant to heat than the agglutinin in the sera of rabbits immunized with Proteus X_{19}. But the O agglutinin present in the serum of a patient infected with Proteus was as resistant to heat as that in the immune rabbit serum.

Two interesting papers by Jones (44,45) were published in 1927 and 1928. The first dealt with the effect of heat on these antibodies present in immune rabbit serum: flagellar and somatic agglutinin, red-cell agglutinin, hemolysin and precipitin, All the sera were diluted 1:2 or 1:4 before they were heated. After being heated the different antibodies were entirely inactivated as follows: the somatic agglutinin and precipitin at 75 C, the red-cell agglutinin at 80 C and the hemolysin at 85 C. Some flagellar agglutinin remained even after the serum was heated at 90 C. While the activity of all except the red-cell agglutinin was diminished after 20 minutes at 65 C, it showed a sharp decline at 70 C, a temperature at which the other antibodies were fairly stable. All of the antibodies were unaffected after 8 hours at 50, 55 and 60 C.

All except the flagellar agglutinin were destroyed after longer periods at 60 C.

In the second investigation the inhibiting effect of heated immune sera was studied. The antisera were prepared in rabbits with B.abortus and with a motile strain of hog-cholera bacillus as antigens. With both of these antisera Jones was able to demonstrate a specific inhibiting effect on the unheated sera. The antiserum to B.abortus showed this inhibition after 20 minutes at 70, 75, or 80 C; the other antiserum showed this effect most markedly after 20 minutes at 75 C. Both had neither inhibiting nor agglutinating power after being heated at 90 C. Without giving the details of the experiment Jones said that antisera for B.abortus heated at 70, 75, and 80 C entirely or partially fixed complement. In addition he made the interesting observation that organisms exposed to their homologous heated serum, subsequently removed, and washed with physiological salt solution were agglutinated by antirabbit serum. Thus he showed beyond doubt that inhibiting nonflocculating antibody was able to combine with its antigen.

In experiments designed to find an explanation for the inhibition-zone Shibley (71) utilized the inhibiting effect of heated immune serum. His agglutinating antisera were prepared in the rabbit or in the horse with E.typhosa, B.melitensis, D.pneumoniae, and several of the Shigella as antigens. In all cases the sera were diluted either 1:5 or 1:10 in salt solution before they were heated, because he found that when they were

heated in stronger dilutions or undiluted the sera became turbid. Both the temperature used and the time of exposure to this temperature proved to be important in effecting the phenomenon of inhibition. Different temperatures were required for different sera, the range being from 62 to 76 C with short periods of exposure - 6 to 10 minutes. The point at which all agglutination was abolished ranged from 64 to 78 C. It was possible to produce an inhibition zone by heating for prolonged periods at lower temperatures; for example comparable zones were produced by heating the same serum for 10 minutes at 66 C and for 4 hours at 59 C. In general the time required for the inhibition-zone to appear, to become complete, and for all agglutinating power to be destroyed was more or less proportional to the temperature. An experiment in which antishiga serum was heated for 10 minutes at temperatures ranging from 55 to 76 C showed an interesting and puzzling result: the inhibition-zone appeared at 64 C; widened to reach a peak at 66 to 69 C; became narrower at 70 C and from 71 to 74 C agglutination occurred as it had before the serum was heated with the exception of a slight lowering of the titer.

Another point which Shibley investigated was the effect of the H ion concentration of the diluted serum. With the temperatures from 70 to 75 C and H ion concentration of 5.4 to 7.0 the broader inhibition-zones appeared at the higher pH's.

Shibley confirmed Jones' observation that the inhibition-zone was a serologically specific phenomenon, both by the use

of heated heterologous serum and by showing that homologous serum, absorbed after heating, did not inhibit agglutination by unheated serum. Now that we know more about quantitative relationships existing between antigen and antibody, his observation regarding the influence of the concentration of the bacterial suspension upon the appearance and breadth of the inhibition-zone has added interest.

One of his heated sera developed a faint opalescence which could not be removed in the centrifuge. But after filtration through a Berkefeld N filter this serum no longer had an inhibition-zone showing that there had been physical alteration of the antibody, probably an increase in size.

Coca and Kelly (15) reported the unusual circumstance of obtaining an antiserum for H.influenzae that would not agglutinate its homologous strain but would agglutinate heterologous strains. This nonagglutinating serum was capable of specific inhibition, and it was possible to adsorb the inhibiting substance with the homologous organism. Such absorbed serum would no longer agglutinate the heterologous strains. These authors did not consider this naturally occurring, nonagglutinating antibody modified agglutinin or agglutinoid. All that can be said is that chemically and physically it might be quite different from agglutinin modified by heat or ageing, but reactively, it was similar. We know from more recent work, the presence in serum of combining but nonprecipitating antibody is probably not uncommon in the early stages of immunization. The presence of

such antibody has been demonstrated by Pappenheimer (65) and by Heidelberger and Treffers (37) in the same equine antiovalbumin.

Felton (26) and Felton and Bailey (27) heated equine antipneumococcal sera and antibody-solution to determine the effect of heat on protective power. Type I serum after 60 minutes at 56 C showed only slightly diminished protective power but it no longer precipitated on dilution with distilled water, and according to Felton "...takes on the pearly sheen of an alkaline serum in solution". Six samples of fresh type I antipneumococcal serum heated for 30 minutes at 56 C retained their protective power, while 2 samples of antibody solution under similar conditions lost 20 and 75 per cent respectively of their protective power. The agglutinative, precipitive and complement fixing titers of both serum and antibody-solution dropped markedly after they were heated; the decrease with the exception of the fixing titer was more marked for serum than for antibody solution. Felton and Bailey concluded that the antibodies in antipneumococcal sera, other than the protective one, were relatively thermolabile. Their explanation for the decreased reactivity was the alkalinity of the sera - pH increased by 0.3. Rather than indicating the presence of two or more antibodies with different thermal lability, probably these results indicate one antibody in which the combining power is unaffected by heat and hence the power to protect remained.

The effect of heat upon the ability of a serum to confer passive sensitivity was determined. In the experiments of

Weil (82), which he repeated several times with identical results, rabbit antihorse serum diluted 1:10 in physiological salt solution and heated for 30 minutes at 70 C lost its power to precipitate but its power to sensitize was only slightly diminished. Coca (16) said that he and Grove found anaphylactic antibody slightly affected in sensitizing power by heating for 30 minutes at 56 C.

Finally, there appeared in the German journals several papers presenting the results of experiments, in which substances that prevent the coagulating effect of heat on protein, were used in studying the effect of heat on immune sera.

Silber and Schaecter (72) attributed the loss of agglutinating power in immune serum after it was heated to changes in physical state and not to the destruction of antibody. They said that a pH of 8.3 to 10 or the presence of a substance called fermentoid prevented the coagulation of serum so that the alkaline serum could be heated for one hour at 70 to 96 C and serum containing fermentoid at 70C without lowering the titer. This finding of a favorable effect with increased alkalinity is the reverse of the results of Streng who, according to Marrack (59a), found that the destruction of agglutinins by heat was much accelerated by the addition of alkali.

Silber and Nikolskaja (73) studied the thermostability of agglutinins in the presence of substances that retard coagulation. Heat did not affect agglutinin when Bayer 205 or hydrochloric acid was present in the serum; and the presence of

glycerol, 3 volumes, and of sucrose in agglutinating sera permitted heating for 60 minutes at 75 C with no change in titer.

Another paper by Beilinsson (9) dealt with the effect of heat upon serum and upon egg albumin in the presence of glycerol and sucrose. He found their physico-chemical properties more or less unchanged after being heated under such conditions; the protective action of glycerol was less than that of sucrose and it varied according to the concentration, and the temperature used. In a sufficient concentration of sucrose rabbit serum was completely thermostabile at 62 C and egg albumin at 75 C.

These three papers were read as abstracts and the experimental details were not given, so it is impossible to comment on the results reported by the authors.

During the second period some experimental results reported by earlier workers were confirmed. Investigation of old problems was continued, new facts were discovered so that added information about the effect of heat upon antibody, as it exists in immune serum was obtained. The more important facts ascertained are summarized below.

The existence of somatic and flagellar agglutinins and their different resistance to heat was confirmed.

The combining power of agglutinin modified by heat was confirmed.

Complete destruction of different antibodies was observed when the antisera were heated at 64 to 90 C for 6 to 30 minutes, and the formation of modified agglutinin with the appearance of the inhibition-zone at 62 to 80 C for the same length of time.

It was shown that the concentration of H ions, the concentration of neutral salts, the presence of phenol, and the concentration of the serum - that is, whether diluted or undiluted - are important factors in determining the effect of heat on immune sera.

Concerning the development of inhibition and the inhibition-zone the following facts were ascertained: inhibition is specific; longer periods of heating at lower temperatures are as effective in the formation of nonagglutinating antibody and the inhibition-zone as shorter periods at higher temperatures; the pH of the serum heated determines the width of the inhibition-zone; the concentration of the bacterial suspension used in testing the heated serum is a factor determining the width of the zone.

Naturally occurring, nonagglutinating, inhibiting antibody was observed.

The protective power of heated equine antipneumococcal serum is only slightly diminished, but the agglutinative, precipitive, and fixing titers are markedly lowered.

Heated immune rabbit serum that does not precipitate its antigen confers passive anaphylaxis.

The application of heat under controlled conditions is a useful measure in refining some therapeutic sera.

Scientists interested in the chemistry of the proteins have had in recent years the use of two important tools, the ultracentrifuge and the Tiselius apparatus. Ingenious serological investigations, also, have contributed to our present increased, but still inadequate knowledge of these important substances. All advances in our understanding of the structure and properties of proteins have reacted favorably on investigations of antigens and antibodies. Improved methods of analysis for the constituent amino acids in proteins, on which some chemists have worked patiently for years, have justified the time and labor expended upon them. Re-examination of the theory of the structure of proteins by Chibnall (14) has provided a new conception of their structure, and has, perhaps, brought us nearer to an understanding of these complex compounds.

van der Scheer, Wyckoff, and Clark (79) were the first to use the electrophoretic method of Tiselius in the study of heated serum. After normal equine serum had been heated at 65 or 70 C for 30 minutes they observed the presence of a new component which they called C. This component had the following characteristics: (1)the electrophoretic mobility was like that of beta-globulin; (2) it arose at the expense of the globulins and finally the albumin; (3) it was insoluble in distilled water; (4) it was heterogeneous. Albumin and globulin were obtained by half-saturating the serum with ammonium sulphate. The solutions were dialyzed and adjusted to the volume of the original serum. When these solutions were heated separately there was no change

in the electrophoretic pattern of the albumin, but the globulin developed a huge C-peak. The material responsible for this C-peak could be removed by centrifugation under conditions that would not sediment globulins in true solution. A heated, reconstituted serum developed a C-peak that was similar to the one in the heated serum. When tetanal antitoxin was heated a C component developed with a reduction in the albumin, and T and γ components. Heated antipneumococcal serum coagulated after 10 minutes at 65 C; electrophoretic analysis of the supernate revealed that the γ component had disappeared.

The formation of the C component in antipneumococcal serum was observed by Krejci et al (54) after the serum had been heated at 64 to 65 C for 10 minutes. This heated serum retained its specific precipitability - one of two that did so out of the nine examined - and after heating the serum was found to contain 70 per cent more specifically precipitable nitrogen. They were able to sediment this C component by centrifugalizing the heated serum at high speed; the supernatant had a much lower concentration of gamma globulin and no specifically precipitable nitrogen. The redispersed sediment was practically pure C component and most of it was specifically precipitable with homologous polysaccharide.

The finding of Krejci et al (54) that heated antipneumococcal serum contained an increased amount of specifically precipitable nitrogen was confirmed by Hendry and Murdock (38). They noted rather constantly an increase of 1.2 times with no increase in protective power. In addition they made the

interesting observation that pneumococcal antibody of 90 per cent purity, obtained by dissociation from carbohydrate, behaved differently depending upon whether it was heated in saline or in normal equine serum. In the latter case there was an increase in specifically precipitable protein; in the first case there was a decrease in precipitable protein and protective activity.

The experiments of Jennings and Smith (43) and Krejci, Jennings, and Smith (55), in which equine pneumococcal antibody was heated in the presence of nonspecific protein, have provided additional information about the inactivation of antibody. Precipitability of the serum was completely lost after 10 minutes at 65 C but the water-insoluble globulin from the serum heated for the same time and temperature retained its precipitability. The globulin failed to precipitate when it was so heated, if previously it had been mixed with normal horse serum, heterologous immune serum, and the water-soluble proteins of homologous immune serum. Nonprotein colloids, and the following proteins: gelatin, ovalbumin and myogen (rabbit) had no effect upon the antibody when it was heated in their presence. Casein, however, did inactivate the antibody under similar conditions. When the heated mixtures containing casein were examined by the electrophoretic method it was found that the antibody-globulin had formed a complex with the alpha casein. Antibody-globulin of unchanged mobility was present, also. These authors did not test the various preparations of nonprecipitating antibody for their combining or inhibiting properties.

One very interesting short article has been published by Hardt, Huddelson, and Ball (32) in which they described the electrophoretic patterns of unheated bovine plasma, bovine plasma heated for 60 minutes at 65 C, and bovine plasma saturated with glucose and heated for 60 minutes at 65 C. The plasma containing glucose remained clear and did not have a C component in its pattern; it did have a small new peak X in the descending pattern next to the alpha-2 globulin and the fibrogen, beta globulin and gamma globulin areas were decreased. In the plasma heated without the addition of glucose a coagulum formed. The electrophoretic pattern obtained with material cleared by spinning showed the C component.

Papers by Kleczkowski (50,51) and by Bawden and Kleczkowski (7,8) have dealt with the effect of heat on immune rabbit sera. In one of these Kleczkowski presented the results of experiments with antisera for a number of different antigens some of which formed precipitates of the H-type and some of the O-type; the the tobacco mosaic virus was used to exemplify the first type and the tomato bushy stunt virus the second. Some effects resulting after these two sera were heated are shown below. The sera were diluted 1:10 in saline before they were heated.

	Tomato Bushy Stunt		Tobacco Mosaic	
Temperature	Precipitation	Inhibition	Ppt'n	Inhibition
10' 75 C	0	+	+	0
10' 80 C	0	++	+	0
10' 90 C	0	0	0	0

It is evident that the antibodies for both antigens were destroyed at 90 C, but at 75 and 80 C these antibodies behaved in a directly opposite way with regard to precipitive and inhibitive properties. When weaker dilutions of the bushy stunt antiserum were heated, the precipitating power was less affected and the phenomenon of inhibition did not appear. When a solution of rabbit serum-albumin was used as a diluent for both sera the destructive effect of heat on precipitation was increased and the inhibitive power of the bushy stunt antiserum was enhanced.

The antibody in these antiviral sera was separable in the euglobulin-fraction. The effect of heat - 70 C for 15 to 240 minutes - upon solutions of this redissolved protein was determined. In comparison with whole serum there was no loss in precipitating power of the antibody for the bushy stunt virus and the antibody for tobacco mosaic was less affected than it had been in whole serum. When these antibodies were heated in the presence of albumin the results were similar to those obtained with whole serum. The two antibodies, therefore, reacted differently to heat only when the antibody-containing globulin was heated in the presence of other proteins.

Bawden and Kleczkowski (7) obtained some interesting results when they heated various antigens in saline, and in a solution of rabbit serum-albumin. These are shown below.

Diluent	Antigen	Reaction with antiserum
Saline(pH 7.0)	bushy stunt virus	flocculation more rapid and in higher dilution
Albumin	"	combination but no flocculation
Saline(pH 7.0)	tobacco mosaic virus	large loss in serologic activity
Albumin	" "	slight change in " "

Both of these viruses after being heated in the presence of human serum-albumin were precipitated by an antiserum for this protein. But human serum-globulin after being heated in the presence of either rabbit or human serum-albumin, and human serum-albumin in the presence of rabbit serum-albumin, combined with but did not precipitate with their specific antisera. Thus these experiments show that the plant viruses can form complexes with proteins of animal origin under the influence of heat. Also, that proteins of different animal origin can form complexes when they are heated together.

In a later paper Bawden and Kleczkowski (8) investigated the antigenicity of some of these complexes by injecting rabbits with virus and with human serum globulin both complexed with rabbit serum-albumin. The antisera obtained appeared to be identical with those produced by rabbits when normal virus or globulin was used as an antigen. The nonprecipitating complexes fixed complement. These same authors made an extensive study of the effect of heat on the precipitating, fixing, and inhibiting power of antisera to bushy stunt and tobacco mosaic viruses. The results are tabulated below.

	Precipitation	Fixation	Inhibition
At 75 C			
bushy stunt virus	0	+	+
tobacco mosaic virus	+	+	0
At 80 C			
bushy stunt virus	0	+	+
tobacco mosaic virus	0	+	?

They found that the power to neutralize infectivity and to fix complement were equally affected by heat. The antisera for tobacco mosaic virus lost all their serologic reactions almost simultaneously, but the other antisera lost the power to precipitate before the other reactions were affected.

Finally, Kleczkowski (52) determined the influence of various salts in different concentrations upon the formation of complexes when solutions containing proteins were heated. He found that the effect of salts depended upon the salt used and its concentration, and the pH of the solution, - that is, whether it was on the alkaline or acid side of the isoelectric point of the proteins.

There remain to be discussed a few studies that deal with the effect of denaturating agents other than heat upon the serologic activities of immune sera. Neurath and his associates have published many papers dealing with the denaturing effect of heat, urea, and guanidine hydrochloride on proteins; in one of these studies antipneumococcal serum was used. All of the work bears directly or indirectly upon the problem of denaturation of antibody. But, since the related problem of the denaturation of proteins could properly be the subject for a long thesis, it will be possible to mention only a few of their papers.

Neurath, Cooper, and Erickson (61) found that urea and guanidine hydrochloride caused a large increase in the relative viscosity and a decrease in the diffusion constant of solutions of crystalline equine serum-albumin, but the molecular weight of the protein did not appear to change. They thought these changes were due to an increased molecular asymmetry resulting from an unfolding of the molecule. Each concentration of urea or guanidine hydrochloride probably represented a certain stage in the unfolding process. Cooper and Neurath (17) stated that heat effected changes in viscosity, diffusion, and electrophoretic pattern of this same protein. The pH and ionic strength of the solutions were important factors in these changes. After being heated in a solution of pH 7.6, the albumin differed from the native in average particle size and shape, in electrophoretic mobility and pattern, and in susceptibility to tryptic digestion. The changes in mean particle shape at this pH were small in comparison with those produced by concentrated solutions of urea or guanidine hydrochloride, and the changes did not indicate unfolding of the molecule.

A certain amount of reversal of denaturation was possible after the crystalline albumin had been denatured by urea if the urea was removed by dialysis. The regenerated protein resembled the native in size and shape but differed in solubility, electrophoretic pattern and in response to tryptic digestion. Erickson and Neurath (23) concluded that the regenerated protein lacked "...the specific intrinsic configuration of the native protein".

They immunized rabbits with the native and with the regenerated compound. With two exceptions the native stimulated the production of a much larger amount of antibody than the regenerated protein. All the antiregenerated sera had an identical combining capacity for both proteins, but two of the antinative sera were able to combine with 5 to 8 times as much native protein as they were with regenerated protein. They considered these two proteins to be immunologically equivalent. Doubtless they were with some of the sera but certainly not with others. They concluded that the physico-chemical properties which they studied were not important in determining the antigenicity of the native protein.

Neurath et al (62) reported that the effect of urea on equine pseudoglobulin was similar to the effect on the albumin, but the effect of guanidine hydrochloride differed depending upon the concentration.

In a short article in Science, Erickson and Neurath (24) reported one experiment in which guanidine hydrochloride was used to denature antipneumococcal antibody for the type I polysaccharide. The antibody was obtained by dissociation of the specific precipitate. The total amount of precipitable nitrogen was determined for the untreated antibody, for regenerated antibody, and for irreversibly denatured antibody. The antibody-antigen ratios for these three preparations were different; there was a difference too, in the per cent of the total antibody that was precipitable. The authors considered their results did not

support Pauling's hypothesis that the difference between antibody-globulin and normal globulin is merely one of steric arrangement.

The denaturing effect of urea on concentrated diphtheric antitoxin was studied by Wright (84). Pappenheimer et al (66) had reported previously that this antitoxin was not destroyed by strong urea solutions. Wright used urea in concentrations of 1.9 to 7.6 molar allowing the antitoxin to remain in contact with the urea for 48 hours. The urea was then removed by dialysis. One hundred per cent of the flocculating and neutralizing activity remained in some of the samples, but the time of flocculation increased. Sixty percent of the neutralizing power remained in a sample that did not flocculate in the Ramon Test. The H ion concentration was an important factor in the denaturation of the antitoxin by urea; the least effect was found at pH 8. The inactivation of antitoxin at pH 7.82 in a concentration of 7.5 molar urea was found to be a first order reaction with respect to the antitoxin.

Wright explained the denaturing effect of urea on antitoxin on the basis of Pauling's theory of the nature of antibody in that he considered the urea competed for the hydrogen bonding affinity of the peptide linkage and perhaps by breaking other hydrogen bonds. The loss in flocculating power he attributed to the formation of univalent antibody.

There is a fairly extensive literature dealing with the effect of ultraviolet light and photo-oxidation on proteins. A few papers have appeared in which the results of photo-oxidation of antibody were described. Harris (33,34) studied the photo-oxidation of plasma, and some proteins and amino acids. He found that hematoporphyrin, chlorophyll, and certain dyes increased the the velocity of the oxidation of plasma. Although many proteins would take up oxygen when exposed to the radiations of the mercury arc, gelatin did not. Most amino acids were insensitive to these radiations but tryptophane and tyrosine absorbed oxygen at a rapid rate.

Henry (39) investigated the photochemical despeciation of equine serum as a possible solution of the problem of sensitivity to foreign protein. Normal horse serum was irradiated for 3 to 4 days with visible light or with ultraviolet light of known intensity and wave length. Determination of the antigenicty of the treated serum was made using guinea pigs and rabbits. He stated, "fresh antigenicities of deviated specificity and of an activity of the order of 1/50th, 1/100th, and less than 1/20,000th that of normal horse serum were obtained". There was a residual content of material having the same antigenic activity as normal equine serum approximately equivalent to 1/1000, 1/10,000, and 1/100,000 that of the untreated serum. There is much interesting material in the discussion-section of this paper that it is impossible to consider in detail.

Ross carried out experiments to determine the photodynamic action of methylene blue and light on antipneumococcal serum

(69), and on diphtheric antitoxin (68). These two sera were affected differently; contrary to the usual experience in studies of denaturation, the antipneumococcal serum was the more resistant. In the case of the antitoxin both the neutralizing and flocculating power was lost. The antipneumococcal serum showed reduced precipitating, agglutinating, and protective powers. However, it would combine with its antigen even though it was altered. When this latter serum was diluted in normal serum the loss of agglutinating power was less than it was when the serum was diluted in saline and exposed to the photodynamic action of light and methylene blue.

In the same year that Ross published his papers, a short one on the same subject appeared by Zia, Chow, and T'ung (83). They used antityphoid serum, antipneumococcal serum type I, diphtheric antitoxin, and purified antipneumococcal serum type I. The dyes used, eosin and methylene blue, were found to have similar action although the optimal concentration was not the same for both. The titers of the H and O agglutinins in the antityphoid serum were much reduced; the reduction was greater for the O agglutinin. The neutralizing power of the antitoxin was reduced, and, also, the amount of specifically precipitable nitrogen obtained from the antipneumococcal serum.

Recently Tyler (76,77) and Tyler and Swingle (78) have published papers dealing with the photo-oxidation of sera. In the first of these Tyler used agglutinating and precipitating sera prepared in rabbits. The agglutinating and precipitating power

was destroyed, but the ability to combine specifically, - as shown by inhibition of untreated sera - was retained. The altered antibody was nondialyzable and could be salted out at one half saturation with ammonium sulphate. Separation of this fraction with ammonium sulphate, followed subsequently by irradiation showed that the agglutinin was resistant to exposures 3 to 4 times those required to inactivate whole serum. In the second paper further experiments with rabbit serum were presented, and some experiments with diphtheric antitoxin. Antiserum for sheep erythrocytes prepared in the rabbit and photo-oxidized to the point where it had a maximal inhibitive titer showed a 50-fold reduction in its direct anaphylactic action on guinea pigs. Two photo-oxidized rabbit antipneumococcal sera that would not precipitate, but would inhibit, had progressively decreased ability to confer passive sensitization in guinea pigs. The protective value of the sera was reduced one half and one twelfth respectively. Photo-oxidation of diphtheric antitoxin resulted in a 50-fold reduction in its power to shock guinea pigs actively sensitized with untreated serum, but in only a 2-fold reduction in its capacity to neutralize toxin.

In a third study by Tyler and Swingle (78) the protective value of photo-oxidized antipneumococcal rabbit serum and diphtheric antitoxin was determined. The antipneumococcal serum, photo-oxidized to the point where it would no longer precipitate with its antigen,had approximately one half its protective power for mice. With further treatment the protective value

dropped off rapidly to zero while the inhibiting titer passed through a maximum. By salting out with ammonium sulphate and by electrophoresis they were able to separate a precipitating and protective antibody from a treated serum. The diphtheric antitoxin was more resistant to photo-oxidation. After it became nonflocculating, it had the same antitoxic value as the control. As photo-oxidation was continued the antitoxic value was slowly reduced.

Tyler, and Tyler and Swingle explained all their results on the premise that univalent antibody was produced as a result of photo-oxidation of the immune sera. In the sera subjected to electrophoretic analysis they observed an increase in mobilities and a decrease in the number of components. This finding was considered indicative of an interaction of the serum proteins.

A paper by Smetana and Shemin in which they reported the results of studies on photo-oxidation of antigen and antibodies was overlooked when the bibliography was arranged. This paper appeared in the Journal of Experimental Medicine in 1941, volume 73, pages 223 to 242. The antisera studied were rabbit anti-ovalbumin and the gamma globulin from this serum, equine anti-pneumococcal serum and Felton's antibody solution. Hematoporphyrin-HCl was used as the sensitizer. As photo-oxidation was continued the amount of specifically precipitable nitrogen decreased and less antigen was required to produce the maximal precipitate. The sera were neither tested for inhibitive properties, nor for combining power after the ability to precipitate

was almost gone. In an attempt to find an explanation for the altered reactivity of the sera the per cent of tyrosine, tryptophane, cystine, and noncoagulable nitrogen was determined for untreated and for photo-oxidized Felton antibody-solution and for the gamma globulin obtained from the rabbit anti-ovalbumin serum. There was no change in the amino acids. The noncoagulable nitrogen was slightly increased in the case of the antibody solution. The per cent of Non coagulable nitrogen was doubled in the solution of gamma globulin, but Smetana and Shemin did not consider this significant. They thought splitting of the molecules was not indicated. Photo-oxidation caused a marked alteration in the electrophoretic patterns of the sera, and when these sera were spun in the ultracentrifuge there was an increased sedimentation rate indicating the formation of aggregates.

The discussion of the results obtained in this period by investigators using different methods of denaturating antibody will follow the section on experimental work.

EXPERIMENTAL WORK

The object of the experimental work was to determine the effect of moderate heat on the flocculating and combining properties of antihemocyanin. Most of the experiments were carried out with an equine antihemocyanin obtained from a horse that had received a series of injections of the hemocyanin obtained from the blood of Busycon canaliculatum. The fifth bleeding was used in all the experiments with this serum. Rabbit antihemocyanin

was used in a few experiments. The hemocyanin used as antigen was obtained by isoelectric precipitation from the blood of Busycon; it was twice reprecipitated making a total of three precipitations.

Preliminary experiments, carried out in 1940, showed that the equine antihemocyanin after being heated at 67 to 70 C for 20 to 30 minutes would no longer flocculate when mixed with varying amounts of hemocyanin. However, the heated antiserum would inhibit markedly and specifically the flocculation of unheated serum when it was present in an optimally flocculating mixture. Flocculation was delayed for many minutes up to three or four days. The mixtures containing heated antiserum gave more precipitate than the control-mixture.

Between November 1940 and March 1944 six different samples of equine antihemocyanin were heated for 20 or 30 minutes at 68 to 70 C. After they were heated, all except sample #6 were tested for flocculative, inhibitive and additive properties. The typical procedure is shown below. The most rapidly flocculating amount of antigen for 0.5 ml of a 1:5 dilution of the unheated serum was determined, and this amount of hemocyanin contained in 0.5 ml of saline was used in testing the inhibiting power of heated serum.

Inhibition shown by heated sample #1

Unheated 1:2.5	ml	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	-
Heated 1:2.5	ml	0.25	0.22	0.20	0.17	0.14	0.12	0.10	0.05	-	0.25
Sol. of Antigen	ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time in minutes							300	108	60	15	

*Volumes were adjusted to 0.5 ml with salt solution before the dilution of antigen was added.

After 48 hours the remaining tubes, with the exception of the tube containing heated serum only, contained flocculent precipitate.

Samples 1 to 5 did not flocculate with antigen after they were heated, and all of them when tested as shown above delayed or completely inhibited flocculation of unheated serum and hemocyanin. Those tubes in which flocculation occurred after the mixtures had stood for 24 hours or longer contained larger amounts of precipitate than the control to which no heated serum was added. The different samples showed some variation in the degree of their inhibitive and additive properties. They were the least marked in sample #3. This sample was heated in an amount of 1000 ml while the others were in amounts of 30 to 100 ml. It is possible that this larger volume did not attain a temperature of 68 to 70 C throughout. As the serum aged it tended to become more Viscid when it was heated, and tended, also, to lose rather quickly the inability to form precipitate with antigen.

The amount of nitrogen present in washed precipitates obtained from various mixtures of unheated and heated serum and

antigen was determined by the microkjeldahl method. Samples 1 to 5 were tested for additive effects in a mixture containing optimal quantities of unheated serum and antigen, samples 1 and 2 for additive effects in a mixture containing an excess of antiserum, and samples 2 and 4 for such effects in a mixture containing an excess of antigen. The uniform procedure used in preparing the mixtures was to pipette into the tube the amounts of heated and unheated serum, then the hemocyanin, and finally saline in sufficient amount to bring the contents of each tube to the same volume. Table I shows in detail the results of such experiments with sample #2. Inspection of this table shows that the presence of 1/4 to 1-1/2 volumes of heated antiserum in the mixtures caused about the same increase in the precipitate in each zone as shown by the amount of nitrogen present. The presence of heated normal equine serum in different mixtures of unheated antihemocyanin did not increase the amount of precipitate formed. The same result was obtained with heated diphtheric antitoxin in the optimal zone; it was not added to the other mixtures. In one instance a mixture of heated serum and hemocyanin was prepared, and the unheated serum was added after the mixture had stood for 48 hours in the icebox. There was no precipitate in the mixture before the addition of unheated serum. The amount of precipitate obtained following its addition was the same as that obtained from a mixture prepared in the usual way.

Table II shows the increase in the amount of precipitate obtained with samples 1 to 4 of heated antihemocyanin when they

TABLE I

SPECIFICALLY PRECIPITABLE NITROGEN OBTAINED FROM MIXTURES CONTAINING BOTH UNHEATED SERUM AND HEATED SAMPLE #2

Optimal Zone

633_5 ml	633_5 heated dil.1:4	Antigen-N μg	Ppt-N μg	Increase
0.16	0.16	100	492	1.4
0.16	0.32	100	570	1.6
dil.1:2				
0.16	0.32	100	320	1.8
0.16	0.48	100	353	2.0
0.16	0.64	100	413	2.3

Zone of Antigen-excess

633_5 ml	633_5 heated dil.1:4	Antigen-N μg	Ppt-N μg	Increase
0.12	0.12	250	477	1.3
0.12	0.24	250	621	1.8
	undil.			
0.1	0.1	200	696	2.3
0.12	0.18	250	1026	2.9
0.25	-	500	742	

Zone of Antibody-excess

633_5 ml	633_5 heated ml	Antigen-N μg	Ppt-N μg	Increase
0.4	0.1	140	688	1.4
0.4	0.2	140	864	1.7
0.4	0.4	140	1031	2.1
0.4	0.6	140	1059	2.1
0.4	-	140	500	

177 = μg N expected from 0.08 ml 633_5 + 100 μg antigen-N

TABLE II

THE INCREASE IN TOTAL PRECIPITABLE NITROGEN WITH DIFFERENT SAMPLES OF HEATED EQUINE ANTIHEMOCYANIN

1. In an optimal mixture of unheated serum and hemocyanin
2. In mixtures prepared in the zones of antigen and antibody-excess

Optimal Mixture

Vol. of heated serum	Increase in ppt-N Sample				
	1	2	3	4	1*
1/4		1.4			
1/3	1.6				
1/2		1.6			
2/3	2.3				
aa	3.6	1.8	1.8	2	2.7
1-1/2		2.0			
2x		2.0	2.2	3	5.0
3x			2.4	4	6.0
4x			2.9	5.4	(7.5 (5.2
5x				5.0	
6x				5.3	

*The mixtures were prepared 2 years later than the first. These mixtures were at the antigen-excess end of the optimal zone.

Sample #3 gave same precipitate with hemocyanin alone.

TABLE II (Continued)

Zone of Antigen-Excess (2x)

Vol. of heated serum	Increase in ppt-N Sample		
	2	2*	4
1/4	1.3		
1/2	1.8	1.7	
aa	2.3		2
1-1/2	2.9		
2x			2.1
4x			6.3

*This sample was 1 year old when this mixture was made. At this time other mixtures were made containing 3 to 4 times the optimal amount of antigen. The unheated gave no precipitate with this quantity. There was no marked precipitate until 3x volume of heated was added; with a 4x volume of heated there was a very large increase in the precipitate.

Zone of Antibody-Excess

Vol. of heated serum	Increase in ppt-N Sample	
	1*	2
1/4		1.4
1/2		1.7
aa	0	2.1
1-1/2	0	2.1
2x	0	
4x	0	

*Heated sample 2 years old. There was no precipitate in the control containing in one case unheated and hemocyanin, and in the other heated and hemocyanin.

were present in the different mixtures. Although there were some differences in the increase found with the various samples the trend was the same in all. It was not certain that maximal increase obtainable was obtained; but in some instances there was a leveling off, for example in sample #2. With the exception of #1 there was increased precipitate in all three zones; this sample, which had been heated about two years previous to the time the mixtures were made, did not add to the precipitate in the zone of antibody-excess.

The precipitates formed in mixtures containing heated serum were very soft and hydrophilic in character so they were very difficult to separate and wash satisfactorily. For this reason the determinations of the total nitrogen in precipitates from duplicate mixtures often did not check so closely as is desirable. Even so, the increases were of such an order there could be no doubt that the presence of heated serum had caused a larger amount of precipitate to be formed. In the first experiments the ordinary centrifuge was used in separating the precipitates. Later it was found that the angle centrifuge was much more effective in separating this very hydrophilic material. When some samples of heated serum were diluted in saline and spun in the angle centrifuge the formation of a lower viscid layer was observed. Probably this layer was similar to the gelatinous button described by Krejci, Smith, and Dietz (54), which they obtained from heated equine ~~and~~ antipneumococcal serum when it was spun at high speed.

After precipitation had taken place in a mixture containing optimal amounts of serum and hemocyanin, the supernate was separated in the centrifuge and then removed from the precipitate. This supernate heated for 30 minutes at 68 to 70 C did not cause the formation of increased precipitate when it was present in an optimal mixture of unheated serum and antigen. Also, this heated supernate had no inhibiting properties.

It was assumed, wrongly perhaps, that since the heated serum did not flocculate when diluted and tested in the usual way that it would not yield a precipitate when mixed undiluted with hemocyanin. The finding, that some of the samples yielded specific precipitates after some months of storage in the icebox, indicated that the precipitability of all subsequently heated samples should be determined soon after they were processed. At the time it was annoying to find that as the serum aged the samples subjected to heat appeared to be more precipitable. The increased alkalinity of the stored serum could be a factor in this changed reactivity of the heated samples. After further study it became apparent that this apparent regeneration of the ability to precipitate was an interesting fact.

The results obtained when heated samples #1, #5, and #6 were mixed with antigen only are set forth in table 3. Samples #2 and #4 did not precipitate with an amount of hemocyanin that was optimal for the unheated serum; other amounts were not tried. Sample #3 which was heated in a large volume showed little difference from the unheated in the few mixtures prepared; after a short reheating it behaved like #5 in table III; that is, when

TABLE III

SPECIFICALLY PRECIPITABLE NITROGEN OBTAINED FROM HEATED EQUINE ANTIHEMOCYANIN 633_5

Sample 1
December, 1942*

Serum ml	Antigen-N μg	Ppt-N μg
0.5	150	trace
0.5	250	(728 (880
0.5	500	(1720 (1638
unheated		
0.5	500	869

Sample 6
March 20, 1944*

Serum ml		Antigen-N μg	Ppt-N μg
0.2		140	962
0.4		140	1683
0.6		140	2127
0.4	+	saline	no ppt.
Unheated			
0.5		100	212

Sample 5
March 24, 1944*

Serum ml	Antigen-N μg	Ppt-N μg
1.0	1200	720
1.0	940	650
1.0	625	1725
1.0	350	1850
1.0	310	2425
1.0	160	(1710 (1218

Unheated 633_5
March, 1944*

Serum ml	Antigen-N μg	Ppt-N μg
1.0	2000	2320
1.0	1400	1970
1.0	800	1668
1.0	400	1090
1.0	200	430

Sample 1 was heated on 11/13/40
Sample 5 was heated on 3/18/44
Sample 6 was heated on 3/20/44
*Date on which the mixtures were prepared

the volume of serum was kept constant and the amount of antigen decreased the precipitate increased. It was evident, therefore, that even though some lots of heated serum would precipitate specifically with antigen, the zone where precipitation occurred differed markedly from that of the unheated serum. There was no precipitate when crystalline ovalbumin was used instead of hemocyanin. Perhaps all the samples of heated serum, even the early ones, would have precipitated specifically, if the correct proportions of antiserum and hemocyanin had been determined.

The experiments with heated rabbit serum were not so extensive as those with equine serum. There was no doubt that the heated rabbit serum would inhibit specifically the precipitation of unheated serum, but convincing proof that it increased the amount of precipitate was not obtained.

The results of the experiments can be summarized as follows:

1. Equine antihemocyanin heated at 67 to 70 C for 20 to 30 minutes combined with antigen but lost its power to precipitate with amounts of antigen that were optimal for the unheated serum.

2. Heated serum inhibited for many minutes up to three or four days the specific flocculation of unheated serum.

3. The presence of heated serum in mixtures of unheated serum and hemocyanin caused slight to marked increases in the amount of precipitate that formed. The additional amount of precipitate varied somewhat with the age of the heated sample and with the age of the serum at the time the sample was heated.

4. The increase in precipitate indicated that heating had caused the formation of complexes between antibody-globulin and

serologically inert protein.

5. Heated normal serum and heated heterologous immune serum neither inhibited the formation of nor added to the amount of specific precipitate.

6. Some samples of heated serum appeared to regain the property of specific precipitation.

7. Those samples of heated that did precipitate specifically differed from the unheated serum in yielding the larger amounts of precipitate with the smaller quantities of hemocyanin.

8. Heated rabbit antihemocyanin inhibited specifically the precipitation of unheated rabbit antihemocyanin.

DISCUSSION AND SUMMARY

The results obtained in the experiments with equine antihemocyanin were similar to those obtained by other investigators using different serological systems. There have been several reports of the specific inhibitive effect of heated antiserum (45,50,71). Krejci et al (54) and Hendry and Murdock (38) found an increased amount of specifically precipitable nitrogen in heated antipneumococcal sera. When crude sera were heated or when purified antibody-globulin was heated in the presence of non-specific protein, and then spun at high speeds, several workers obtained evidence of the formation of complexes (51,54,79). Kleczkowski (51), Bawden and Kleczkowski (7) with serological methods and Jennings and Smith (43) with the electrophoretic

method were able to show that proteins of similar or of different origin would form complexes when they were heated together in solution. There was evidence of some selective action because the antipneumococcal antibody would not form complexes with all proteins. The fact that in the case of antipneumococcal serum (38) the amount of specifically precipitable nitrogen increased without a correspondingly increased protective value for the serum, is a definite indication of the serologically inert character of the added protein. By electrophoretic analysis, van der Scheer et al (79) were able to demonstrate the presence of a new component in both heated normal equine serum and in heated immune equine serum. With the same method Ball et al (3) found a similar component in heated normal bovine serum. Cooper and Neurath (17) who worked with a very pure protein, crystalline horse-serum albumin, observed changes in mean particle shape of the molecules after solutions of this protein had been heated. They attributed the change to an aggregation of the molecules of protein.

Thus, the hypothesis that complexes are formed when crude sera are heated or when antibody-globulin is heated in the presence of non-specific protein is supported by the results of quantitative chemical determinations, by serological methods, by electrophoretic methods, and by sedimentation of the complexes by centrifugation at high speed.

Kleczkowski (51) thought the action of heat on crude antiserum involved two changes: first, a stage when active disulphide

groups can be detected and the changed particles form into large complexes; second, a stage of further alteration in which antibody loses its activity. After the first stage antibody retained its combining power for antigen, but whether or not it retained its precipitating power depended upon the other proteins present and the complexes formed after both were broken down. Krejci, Smith and Dietz (54) agreed with Kleczkowski in explaining that the alteration in heated serum was first a denaturing process and second an aggregating process. Possibly Kleczkowski's observations (51) of the importance of the albumin globulin ratio in determining the effect of heat, may explain the different results obtained when sera from different bleedings of the same animal, or when sera from bleedings of different animals are heated. For example, samples of serum from the fourth bleeding of equine antihemocyanin coagulated when they were heated under conditions that did not cause coagulation of samples of serum from the fifth bleeding. Also, it has been reported that some antipneumococcal sera will retain their precipitability after they are heated at 64 to 68 C while others lose it entirely.

It is pertinent at this point to discuss the experiments of Hardt, Huddelson and Ball (32) in which they found that bovine serum saturated with glucose did not develop the C component when it was heated while serum without glucose did so. Relative to this finding are the experiments of Ball et al (3) in which they found that the presence of several sugars including glucose tended to prevent the formation of SH groups when ovalbumin was

denatured by heat. These results suggest that the glucose prevented the breaking down of the proteins in the serum so that the C component, which appears to form as a result of aggrega- of diverse elements, was not formed.

When most immune sera are denatured by heat, by photo-oxidation, and by the action of urea, the power of specific precipitation or agglutination is lost but the power of specific combination is retained. Other demonstrated effects are the formation of complexes due to the effect of photo-oxidation, and an effect on linkages involving S-S or -SH groups in the case of heat or urea. Eagle (19) and Eagle et al (20) made the suggestion that the loss of visible reactivity with homologous antigen in sera treated with formaldehyde or combined with various diazo compounds was due to the formation of highly soluble antibody. He pointed out that antipneumococcal serum treated with an amount of formaldehyde just sufficient to destroy its precipitating properties also made the antibody-globulin more soluble, shown by the fact that it did not precipitate when the serum was diluted with distilled water. He pointed out, also, that Felton and Bailey (27) had found heated antipneumococcal serum did not precipitate when it was diluted with distilled water. It is conceivable that denaturation by the different methods causes a solubilizing effect so that antibody can combine but not show visible reactivity. If the antibody-globulin reacts to form a compound with the more soluble serum proteins, the result would be more soluble antibody. Other possible results of the action of denaturing agents that would make antibody more soluble could

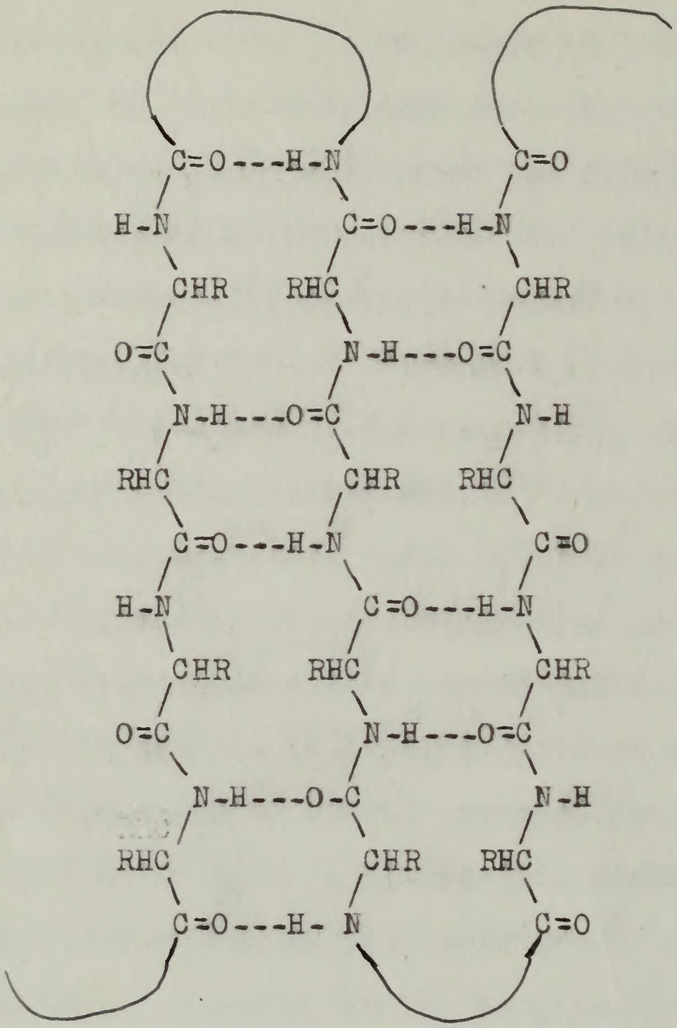
be splitting of the molecule into smaller units, the unfolding of the molecule thus bringing more polar groups into action. As was stated above, the evidence at present indicates that complexes are formed. Besides the factor of the increased solubility of these complexes it is possible that they combine with a smaller amount of antigen due to steric hindrance or to the complete loss of some of the combining sites in the polymerizing process. Only in the case of the photo-oxidation of diphtheric antitoxin (78) has it been shown that nonflocculating antibody retained its full combining capacity. Denaturation by urea (84) resulted in the loss of 60 per cent of its neutralizing power before it became nonflocculating. Inspection of table III shows that those samples of heated antihemocyanin that did form precipitates with antigen had a much altered combining capacity. Regenerated antipneumococcal antibody-globulin, which had been denatured by guanidine-HCl (24), had a much increased antibody-antigen ratio with only 10 per cent less precipitable nitrogen than the undenatured preparation. This indicates a reduced combining capacity for antigen.

It is generally recognized that when proteins are denatured by heat,reactive SH groups can be detected. When urea is used as a denaturing agent there is some change involving the S-S or -SH groups, and in some cases, for example ovalbumin, when denaturation is effected by photo-oxidation. The idea that these linkages are important in the immunological behavior of proteins was stated by Blumenthal (11) who used crystalline serum-albumin

(equine) and crystalline ovalbumin. She stated "...either that the S-S and -SH groups may be among the specific chemical groups involved in antigen-antibody combination, or that the reduction of the S-S linkage may split the antigen molecule into units which are too small to give the same immunological response as the original protein". It has been suggested by others (14,84) that the S-S or thiol-ester linkage are important in maintaining the configuration of the native protein. Smetana and Shemin (p.33) could not find any detectable difference in the S-S and -SH groups in photo-oxidized antibody although others have considered that changes did occur in them when proteins were photo-oxidized. They did not find any change in the content of tyrosine tryptophane contrary to results reported by others when protein was photo-oxidized. These authors thought alteration of antigenicity due to photo-oxidation was not selective but incidental. The best explanation of the formation of SH groups and their relation to the structural integrity of native protein has been offered by Chibnall (14) in his analysis of the structure of protein in the light of recent work, especially the more accurate determinations of the amino acid content of certain proteins. He thinks that the thiol-ester linkage, R.CO.S.R', is one of the linkages binding the units of the protein molecule. Hydrolysis of this linkage would result in the formation of SH groups. His ideas of protein structure will be discussed in detail later. He thinks hydrogen bridges could hold together some of the subunits.

The importance of the hydrogen bond was stressed earlier by Pauling (37), and his conception of the structure of protein will be considered first. He said that a stable configuration of protein would result from the presence of as many strong hydrogen bonds as possible. According to his concept, the molecule of protein consists of parallel layers of polypeptide chains in which the hydrogen bond is important in holding together the parallel chains of a layer, as shown in the diagram on the opposite page. The protein would be composed of several such layers parallel to one another, as he said like a stack of pancakes, these stacked layers being held together by side-chain interactions. According to Pauling it is the configurations of the ends of the chains of antibody-globulin that are important in its specific action. In considering the effect of denaturing agents he said there was one stable configuration of the central part of the molecule, so that it was probable the more labile characteristic configurations at the ends of the molecule were destroyed before the central part of the molecule was affected. It should be stated here that available evidence indicates quite a lot of alteration of the antibody-globulin occurs before its specific combining capacity is lost. Also antibody as it occurs in serum loses first, only its ability to show visible reaction with specific antigen and not its capacity for specific combination.

Chibnall's conception of the structure of protein will be considered and compared with Pauling's conception. Both accept



The folding of polypeptide chains into a layer held together by imino-carbonyl bonds.

(Pauling, L., 1940 J.Am. Chem. Soc., 62, 2643)

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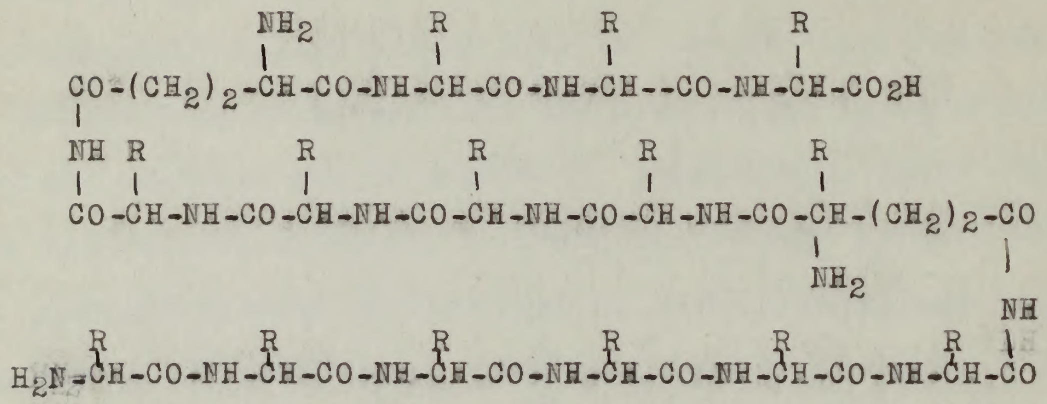
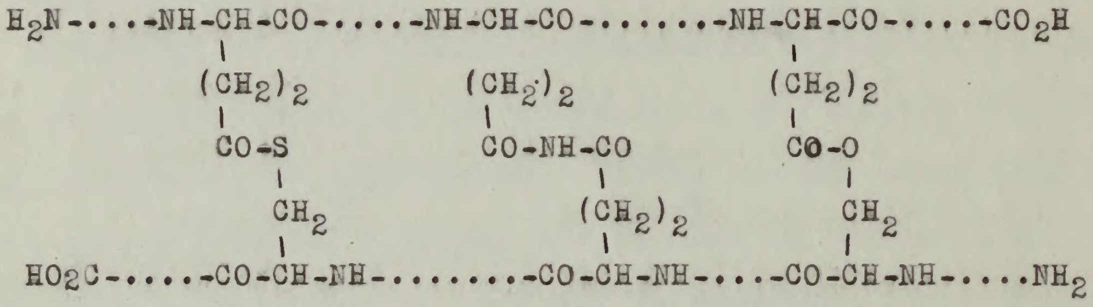
(A.C. Chibnall 1942 Proc. Roy. Soc.,B, 131,136)

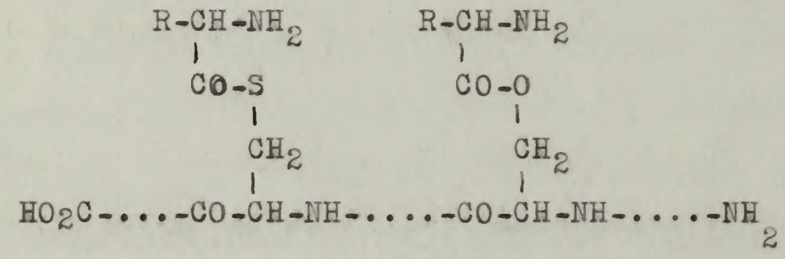
Three peptide chains coupled through terminal linkages

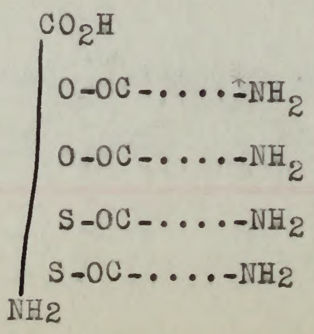


Examples of lateral thiol-ester, imide and ester cross-linkages



Examples of systems with branch-chain linkages





the idea of several chains, made up of a number of amino acid residues, that are held together by some sort of linkage. Pauling emphasized the importance of the hydrogen bond. Chibnall said that this could be one means of maintaining the structure, but he has offered several other possible explanations of the way in which the polypeptide chains are arranged in the molecule.

In his paper Chibnall considered in detail the structure of edestin, β-lactoglobulin, ovalbumin, and insulin. He said that these proteins contained 6, 9, about 4 and 18 polypeptide chains, respectively. Regarding edestin, he said the following: (1) All the constituent peptide chains are of like composition (2) It contains 432 amino acid residues or a multiple thereof (3) The smallest possible molecular unit has a molecular weight of 50,000. In support of the figure 50,000 as the molecular weight of one of the six similar constituent units he cited the apparent molecular weight of 49,000 observed by Burk and Greenburg in a 6.66 M solution of urea. This would indicate he said that denaturation caused a breaking of the molecule into its six component units, and the molecular weight of 300,000 computed from the velocity of sedimentation and the measurement of diffusion supports this idea.

In his analysis of the structure of β-lactoglobulin he has postulated different ways in which the constituent units could be held together in the molecule. In this protein the eight or nine peptide chains present were not considered identical. The various linkages suggested are shown on the opposite page. The

linkages suggested are: the ester linkage, R.CO.O.R', the imide linkage, R.CO.NH.CO.R', the thiol-ester linkage, R.CO.S.R'. If these linkages are present in protein between terminal residues, the arrangement would be as in figure 1. If they are present as cross-bridges leaving the chains with free terminal groups, the arrangement would be as in figure 2. If the linkages are side-chain bridges of the ester and thiol-ester type, the arrangement would be as shown in figure 3. Such a link could consist of one or more residues. He pointed out that the data for lactoglobulin fail to satisfy the Bergmann-Niemann hypothesis and the reason might be because each chain does not contain the same number or sequence of residues. He thought the hydrolysis of the thiol-ester linkage provided a more satisfactory explanation of the appearance of SH groups when protein is denaturated than the supposition that these groups are the result of actual cleavage of the S-S linkage due to reduction.

It is interesting to consider some of the reported findings in denaturation of proteins and antibody with regard to Chibnall's conception of proteic structure. When Neurath, Cooper and Erickson (61) denatured equine crystalline serum-albumin they found that the ratio of the axes of the molecule considered as a prolate ellipsoid changed from 5.0 to 16.8 in 8 M urea, and from 5.0 to 20.9 in 8 M guanidine-HCl. Changed ratios were observed, also, by these authors for equine pseudoglobulin when these denaturating agents were used. They thought that the molecule gradually unfolded in the presence of the denaturing agents with the extent of the unfolding dependent upon the

concentration of urea or guanidine-HCl. However, the molecular weight remained unchanged, which does not indicate depolymerization of the molecule. The question arises whether proteins having the structure discussed above could unfold if the cross-linkages between the peptide chains were broken and the molecule flattened out. Such a change would certainly alter the ratio of the axes. Neurath et al (62) fo~~rme~~uNd evidence that in 3 M guanidine-HCl the molecule of pseudoglobulin split in halves as it unfolded. Since the ratio of the axes returned to very near that of the native protein when the denaturing agents were removed, it is necessary to assume some degree of refolding or re-assembling of the peptide chains in the regenerated protein. When Cooper and Neurath (17) denatured the serum albumin with heat, they thought the changes in mean particle shape did not furnish evidence of the unfolding of the molecule. But there was evidence of fragmenting, since electrophoretic analysis of the heated solution revealed the presence of two components although the solution of native protein had contained only one. Depending upon the pH of the solution there was evidence of aggregation of the molecules with an increase in mean particle size. Thus these results are evidence of a breaking down of the molecule and a repolymerizing of some of them into larger molecules.

If a molecule of protein can unfold as a result of the hydrolysis of cross-linkages and, also, refold, if it can break into separate units that re-unite, there are possibilities for different structural patterns in the molecule after it has been denatured. It is possible that the specificity of antibody-

globulin depends both on a certain composition and sequence of of the amino acids in the peptide chains, and upon the arrangement of these chains in the native protein. If these chains are not broken during denaturation, the order and number of the amino acid residues would remain unchanged. In repolymerization of the chains resulting in increased molecular size, some of the combining sites may be destroyed or become unavailable due to steric hindrance. There is evidence to show that regenerated antibody, which was precipitable to within 10 per cent of the amount of the native antibody, combined with less antigen.

Probably linkages other than the thiol-ester one are broken during denaturation, but the breaking of this one is easily detected. If it is true, as Henry (39) has cited from the work of others, that the effect of ultraviolet radiation and photo-oxidation is to rupture links in the peptide chains adjacent to aromatic residues, it is possible to think of the molecule being sliced parallel to its short axis without breaking of cross-linkages. This would explain the splitting off of fractions of low molecular weight. According to this author's findings, even though fragments were split from the molecule, the result of ultra centrifugation suggested increased size of the protein due to aggregation.

In conclusion it is possible to say that there is evidence of fragmentation and of aggregation of molecules of protein in the various methods of denaturation.

CONCLUSIONS

Some antibodies when denatured by heat, by urea, and by photo-oxidation retain the ability to combine specifically, but lose the ability to precipitate or agglutinate their antigens.

Antisera that can no longer precipitate specifically after they are heated can confer passive anaphylaxis.

The protective power of heated antipneumococcal serum is slightly reduced, but the agglutinative, precipitive and fixing titers are markedly lowered.

The temperature at which the antibody in immune sera is destroyed has been found to vary from 64 to 90 C for short periods of heating.

The formation of altered but combining and inhibiting antibody occurs when immune sera is heated at 62 to 80 C for short periods or after it is heated at lower temperatures for longer periods.

The pH of the heated serum is one factor that determines the width of the inhibition-zone.

The destruction of antibody by heat, like the denaturation of ~~antibody~~ protein by heat appears to follow a unimolecular course.

Factors that are important in the denaturation of antibody by heat are the following: the presence of non-specific protein, the H ion concentration, the concentration of neutral salts, and the concentration of the serum.

Diphtheric antitoxin denaturated by urea retains some of its neutralizing capacity after it no longer flocculates.

When immune sera are photo-oxidized inihibiting power

develops, but the power to shock passively as an antibody or actively as an antigen is reduced. At the point of maximal inhibiting power these latter properties are practically destroyed.

Photo-oxidized rabbit antipneumococcal serum that inhibits but does not precipitate has reduced protective power. With further treatment the protective capacity disappears but the inhibiting titers pass through a maximum.

The neutralizing property of diphtheric antitoxin is more resistant to photo-oxidation than the protective property of antipneumococcal serum.

In the denaturation of immune sera by heat, by urea, and by photo-oxidation there is evidence for the formation of complexes between antibody-globulin and the non-specific proteins in serum.

It is probable the effect upon antibody in immune serum that is common to denaturation by heat, by urea, and by photo-oxidation is a solubilizing effect.

Denaturation of protein, and perhaps of antibody, by heat and by urea probably has the common effect of altering the sulphur linkage.

One characteristic of denatured antibody that retains its power of specific precipitation is an altered antibody-antigen ratio.

The increased amount of specifically precipitable nitrogen obtained from heated equine antisera is due to the complex formed between antibody and non-specific protein.

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ABSTRACT

Studies dealing with the effect of heat upon immune sera can be divided conveniently into three periods. The first period extended from the late eighteen nineties to about 1910. This was an exploratory period in which some preliminary studies of the denaturing effect of heat were made. The following facts were reported: (1) Antibodies can be destroyed by heat but the critical temperature is not the same for all antibodies (2) Antibodies present in the different fractions of serum appear to differ in their thermal lability (3) Species differences may exist with regard to the resistance of antibody to heat (4) Normal and immune antibodies may differ in their lability to heat (5) Agglutinoids and precipitoids are formed when immune sera are heated (6) Flagellar and somatic agglutinins can be differentiated by their resistance to heat (7) Certain substances, for example, urea, that prevent the coagulation of protein will prevent destruction of agglutinins (8) In many instances the destruction of antibody by heat occurs as a unimolecular reaction.

The second period in the investigation of the denaturing effect of heat upon immune sera extended from 1910 to 1935. The scope of the investigations was greater than it was during the earlier period. Some of the earlier observations were confirmed and added information was acquired. Many factors of importance in the concentrating and purifying of therapeutic sera by the

aid of heat were determined, such as, the proper temperature, the time of heating, the pH of the serum and the concentration of neutral salts. There were many investigations of the effect of heat upon flagellar and somatic agglutinins. The phenomenon of inhibition was the subject of one or more thorough studies. Conclusive evidence was presented of the specific character of inhibition by heated immune sera. Also, it was shown beyond doubt that heated nonagglutinating sera combined specifically with their antigens. The presence of inhibiting but nonagglutinating antibody was reported in a native immune serum. It was found that some heated sera retained their protective and passively sensitizing properties after the precipitating or agglutinating property was lost. Toward the end of the period substances like urea, glycerol, sucrose that prevent the coagulation of protein by heat were studied for their effect upon the denaturation of immune sera by heat. They had a protective influence on the antibody when they were added to immune sera before the sera were heated.

During the last few years the understanding of the denaturation of antibody by heat, and by other methods has been furthered by increased knowledge of the properties of proteins. Serological studies, electrophoretic studies, and studies in which the ultracentrifuge was used have contributed to our knowledge of antibody. Utilization of all these methods has brought out the following facts: (1) In some immune sera denatured by various

methods the antibody-globulin forms complexes with the non-specific protein and loses its power of precipitating or agglutinating its antigen. Other serological properties such as the power to combine specifically, to inhibit, to protect, and to neutralize toxin are retained to varying degrees depending upon the extent of the denaturation.

The experimental work presented confirmed the results of others in providing evidence of the formation of complexes between antibody-globulin and serologically inert protein.

A possible explanation of the effect of denaturation in the light of recent concepts of the structure of protein was presented.

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